BioDiscovery

Of mice and men – differential mechanisms of maintaining the undifferentiated state in mESC and hESC

Borislav Arabadjiev[1,2], Rumena Petkova[2], Albena Momchilova[3], Stoyan Chakarov[1*], Rumen Pankov[1*]

1 Sofia University "St. Kliment Ohridsky", Sofia, Bulgaria
2 Scientific Technological Service (STS) Ltd., Sofia, Bulgaria
3 Bulgarian Academy of Sciences, Institute of Biophysics and Biomedical Engineering, Sofia, Bulgaria

Abstract

The persistence of the defining characteristics of undifferentiated cells *in vivo* and *in vitro* is maintained via a complex interplay of several mechanisms, employing molecular events internal to the cell as well as signals originating outside the cell. The exogenous and the endogenous mechanisms maintaining stemness qualities of the cell are intricately interwoven with one another and susceptible to cross-interference. Mice and rats as animal models are almost universally considered to be close enough to humans so as to be used in research and applications eventually intended for use in human biology and medicine, at the same time being related distantly enough from primates so as not to overstep ethical boundaries. Studying the specific molecular features of both species in the context of maintenance of the undifferentiated state of murine embryonic stem cells (mESC) and human embryonic stem cells (hESC) can provide researchers with an unique opportunity to unravel the network of interactions which take part in the decision about cell fate under different conditions; to glean interesting insights into the parallel evolution of the two species and to observe how different variants of basic cellular processes have been tried and tested in the evolutionary process. This review article summarises the basic signalling pathways responsible for the maintenance of the undifferentiated state in mESC and hESC and analyses some specific aspects of the molecular physiology that are unique to the particular species.

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Markers of undifferentiated state of ESC – what's inside and what's outside

> ....man's dominion
> has broken Nature's social union.
> Robert Burns, "To a Mouse, on Turning Her Up in Her Nest with the Plough" (1785)

An almost universally accepted definition of stem cells is: undifferentiated cells that are capable on one hand of renewing their own population and on the other hand of producing differentiated progeny. The former is a basic feature of the undifferentiated state common to stem cells and cancer cells, the latter is a defining 'stemness' characteristic. The means by which an undifferentiated cell (cancerous or non-cancerous) maintains its defining properties is based on complex interplay of several mechanisms, employing molecular events internal to the cell as well as signals originating outside the cell. These signals are recognized, amplified and relayed so as to activate the relevant downstream cellular processes that would maintain the undifferentiated state. The exogenous and the endogenous mechanisms responsible for the maintenance of the stemness qualities of the cell are intricately interwoven with one another and susceptible to cross-activation.

The undifferentiated state is peculiar in a variety of manners, as it requires that the cell suppresses possible differentiation pathways, at the same time keeping them primed and alert so that one or the other could be activated at short notice. It is a delicate balance that may easily be tipped in one direction or another, using exogenous as well as endogenous means of activation. For example, increasing the expression levels of Oct-4, one of the essential 'stemness' proteins by 50 % in murine embryonic stem cells (mESC) induces their differentiation into extraembryonic endoderm and mesoderm, while a 50 % decrease in the level of expression of Oct-4 would result in differentiation into trophectoderm [1]. Similarly, treatment of mESC with various uncomplicated and commonly used exogenous agents, such as retinoic acid; 1 % ascorbic acid with or without DMSO; cyclosporine, etc. would trigger differentiation of mESC into cardiomyocytes [2-4]; while leukaemia-inhibiting actor (LIF), another exogenous factor, is believed to exert a short-lasting inductive effect triggering cardiomyocytes differentiation in very early murine embryos (naïve mESC, see below), but would suppress mesoderm formation and progression to the cardiogenic lineage if introduced at later stage (in primed mESC, see below) [5, 6]. Apparently, the maintenance of the undifferentiated state and the decision for differentiation into any of the possible different cell types is a matter of quantitative and temporal distribution rather than a simple presence or absence of a set of pluripotency factors.

Markers for pluripotency pertaining to mESC and hESC

> *MOUSE, n.*
> *An animal which strews its path with fainting women.*
> Ambrose Bierce. In: The Cynic's Word Book (1906)

There are several endogenous markers of the undifferentiated state that are common between mESC and hESC. This is only natural, as the initial stages of embryonic development of all mammals share many common features and the relevant basic molecules exhibit a high degree of homology. One of the hallmarks of an undifferentiated state both in mice and in men is the expression of the *Pou5f1* (*POU5F1* for the human homologue) gene, coding for the transcription factor Oct-4 (Oct-3, Oct-3/4). Virtually all Oct-4 transcripts in mammalian zygotes originate from the oocyte, and maternal and embryonic transcripts co-exist throughout the early stages of embryonic development [7, 8]. Maternal Oct-4 transcripts begin to decay at stage two blastomers and as the stage of four blastomers is reached, the expression of Oct-4 transcripts of embryonic origin intensifies. By the stage of compaction, all blastomers express Oct-4 at high levels. After the blastocyst is formed, however, the level of expression of Oct-4 in the trophectoderm declines, while the cells forming the inner cell mass (ICM) sustain their high levels of expression of Oct-4 [9, 10]. Both mESC and hESC express Oct-4, though, as we already noted, the maintenance of the undifferentiated state is dependent on a very delicate balance of the actual levels of Oct-4, which is dependent on feedback from other factors characteristic of the undifferentiated state [11, 12].

Another marker typical for the undifferentiated state both in the mouse and the man is Sox2 (SOX2 for the human homologue), a transcription factor expressed in the ICM of mammal embryos [13]. It is believed that Sox2 binds its target sequences in complex with Oct-4, producing conformational changes which enable binding of additional regulatory factors [14]. The Sox2-Oct4 complex plays a major role in the specification of the first distinct cell lineages in the mammalian embryo, the three germ layers [10]. Suppressed expression of murine and human Sox2 alike results in very low levels of Oct-4, which means that the undifferentiated state of Sox2-deficient ESC cannot be maintained [15].

Nanog (NANOG for the human homologue) is the third of the basic markers of the undifferentiated state which are common for most mammals. During the embryonic development the expression of Nanog is detected initially in the morula after the stage of compaction, and, subsequently, in the ICM. After the implantation, Nanog expression persists only in selected regions of the epiblast and the primordial germ cells. Pluripotent cells of murine and human origin alike express Nanog and inactivation of the *Nanog* gene in ESC results in their differentiation along the endodermal lineage [16]. Forced expression of Nanog in mESC renders the cells independent of the presence of some of the exogenous factors, such as the leukaemia inhibitory factor (LIF). Induced expression of Nanog in some types of adult stem cells such as bone marrow endothelial cells can result in reactivation of other stemness genes (such as *Sox2, FoxD3, Oct4, Klf4, c-Myc,* and *β-catenin*), whose expression is usually suppressed in stem cells beyond the pluripotency stage [17].

Markers typical for the undifferentiated state of both murine and human ESC are also alkaline phosphatase, surface antigens TRA1–60 and TRA1–81, and the transcription factor Foxd3 [18-20].

The cells of the inner cell mass of the blastocyst express a panel of surface markers that may be used to distinguish murine ES cells from human ES cells. For example mESC unlike hESC express the stage-specific embryonic antigen SSEA-1, whereas hESC express SSEA-3 and SSEA-4, which are not expressed in mESC [19]. It is notable, though, that human embryonic germinative cells (hEGC) express SSEA-1 as well as SSEA-3 and SSEA-4 [18]. Apparently, the expression of certain endogenous pluripotency markers is type-specific

as well as species-specific.

It has been recently proposed that some types of pluripotent ESC may exist in more than one state with regard to their epigenome, their expression profile, their ability to integrate into foreign cellular environments and certain specificities in the molecular signalling mechanisms responsible for maintaining the state of pluripotency and the differentiation into different cell types. Specifically, some authors favour the concept that some types of stem cells, rodent pluripotent stem cells in particular, may exist either in 'naïve', or 'ground' state or in 'primed' (for differentiation) state. The two states are characteristic for two different periods in early rodent embryo development, naïve mESC being derived from pre-implantation embryos while primed mESC generally originate from post-implantation embryos. Reversion of primed mESC to naïve mESC is possible via introduction of only one exogenous factor (Klf4) [21]. There may be significant differences in the expression profile of various pluripotency markers between the naïve and the primed state of rodent mESC (see below), but the most striking difference between naïve and primed mESC is in their ability to integrate into early embryos, thereby creating chimaeric blastocysts and chimaeric embryos; and to contribute to the germline [6, 22].

As of now, it is unclear whether the naïve state exists in species different from rodents or whether it is unique to rodent ESC only, albeit recently porcine ESC have been derived which were reportedly similar in their properties to naïve mESC [23]. The existence of ground and primed undifferentiated states is still unconfirmed in primate and human ESC, and the existence of more than one state is still subject of dispute [24] as creation of human chimaeras, even at very early embryo stage, is ethically unacceptable. As a workaround, studies have been performed using one of the closest animal models possible – namely, the rhesus monkey. These studies show that rhesus monkey ESC generally fail to produce chimaeric blatocysts, as they are unable to integrate into early embryos, and even when they do integrate, the rates of premature differentiation of ESC and pre-implantation death are massive [25].

Exogenous factors and signalling cascades functioning in the maintenance of the pluripotent state of mESC and hESC

Two old Bachelors were living in one house;
One caught a Muffin, the other caught a Mouse.
Edward Lear, in: "Laughable Lyrics" (1877)

Maintenance of the undifferentiated state of ESC *in vitro* is heavily dependent on exogenous factors. These may be secreted by feeder cells (in case ESC are grown on a feeder layer) or may be added in the growth medium as supplements (when ESC are maintained in xeno-free conditions). The exogenous factors can, in general, be viewed as ligands binding to their respective receptors, thereby activating various signalling pathways. As a result, the expression of various target genes is modulated so as to maintain the stemness state or, alternatively, to trigger different prospective routes of differentiation.

The defining features of basic signalling pathways responsible for the maintenance of the undifferentiated state (and respectively for the exit thereof) in mESC and hESC are presented below.

LIF signalling (JAK/STAT pathway)

Historically, the first mESC cells have been grown in medium conditioned by teratocarcinoma cells [26]. Later, the technique of growing ESC on feeder layers (most often mitotically inactivated mouse embryonic fibroblasts) has been developed [27]. Without the conditioned medium or the feeder layer, ESC always went into spontaneous differentiation which made the researchers assume that the supporting cell type produced and secreted soluble factors in the growth medium which worked to maintain the undifferentiated state of the ESC. As the need for xeno-free ESC increased in the following years, much effort has been put since into characterization of the soluble pluripotency factors, so as to avoid mixing ESC with other types of cells [28, 29]. In 1988, the first of these factors - leukaemia inhibitory factor (LIF) - was identified simultaneously by two independent research groups [30, 31]. Their experiments showed that the addition of LIF to the growth medium allowed mESC cells to continue proliferating *in vitro* without differentiating, in the absence of feeder layer.

LIF is, in essence, a cytokine of the IL-6 family which exerts its effects by binding to a bipartite membrane receptor complex that consists of the LIF receptor subunit (Lifr) and the gp130 subunit [32] (Figure 1).

Murine double mutants of the *Lifr* gene created by targeted gene disruption exhibit severe osteopenia, reduced number of motor neurons and astrocytes and generally do not survive beyond the neonatal period. Similarly, defects in the human homologue of *Lifr* (*LIFR*) result in Stuve-Wiedemann syndrome type 2, a rare congenital condition transmitted in autosomal recessive manner and characterized by bowing of long bones, respiratory distress, feeding difficulties, and episodic hyperthermia which usually results in early neonatal death [33].

Lifr alone has a low binding affinity for LIF, but in its complexed state the binding affinity for the ligand greatly increases. The tyrosine kinase Janus (JAK) is
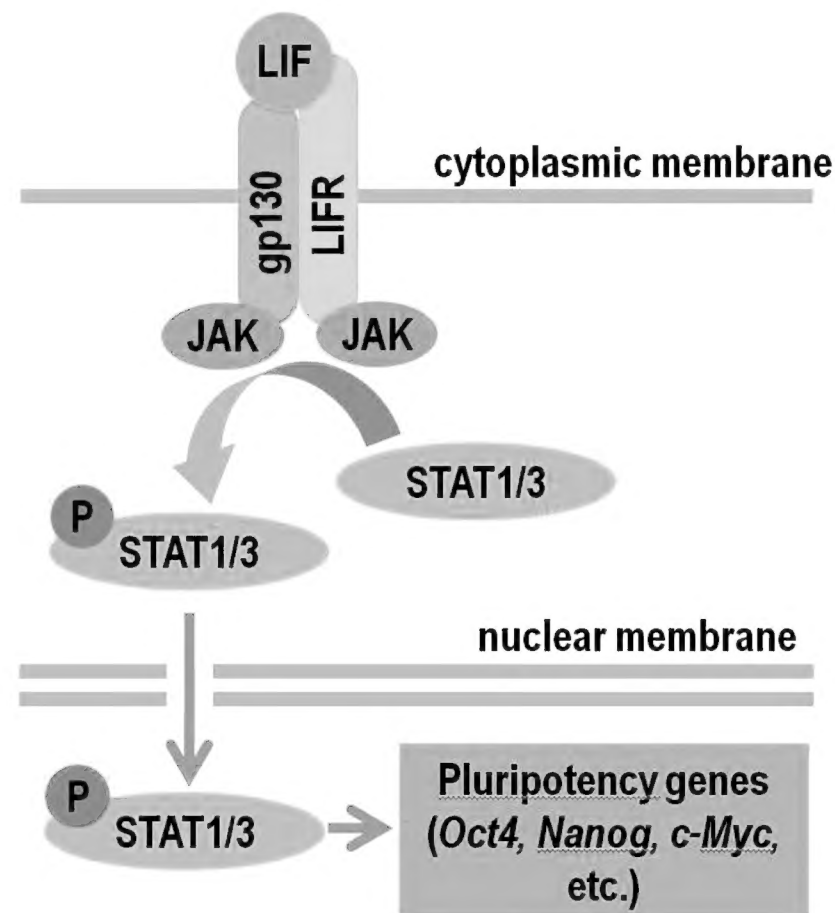
Figure 1. LIF signalling in mESC. Curved arrows indicate phosphorylation, straight arrows indicate activation

constitutively bound to the cytoplasmic part of the complex Lifr/gp130. JAK is activated by binding of LIF to the receptor complex, which results in phosphorylation of specific tyrosine residues of gp130 and Lifr. The latter recruits the transcription factors STAT1 (Signal transducer and activator of transcription) and STAT3 [34, 35]. STAT proteins, in their bound state, are in turn phosphorylated by JAK forming activated homo- or heterodimers. The latter travel from the cytoplasm to the nucleus, where they act to transactivate the expression of other target proteins [36].

STAT3 has numerous target genes. Using chromatin immunoprecipitation (ChIP) in 2008 Chen et al. identified 2546 genomic sites for binding of STAT3, approximately one-third of which (718 sites) were target sites for binding of Oct-4, Sox2 and Nanog [37]. Many of the STAT3 binding sites are in genes directly responsible for the maintenance of the undifferentiated state, including *Oct4* and *Nanog* [38]. Among the target genes of STAT3 in ESC there are transcriptionally active as well as transcriptionally inactive genes. As could only be expected, the transcriptionally active genes targeted by STAT3 generally code for products responsible for the maintenance of the pluripotent state, while the transcriptionally inactive genes are characterized by tissue-specific pattern of expression. Among the latter are *gata* (specific for the ectodermal lineage), *gata4* (endodermal lineage), *lhx1* (LIM homeobox protein 1, mesodermal lineage), *eomes* (trophectoderm), etc. [39].

Among the crucially important target genes activated by STAT3 is the cellular proto-oncogene *c-Myc* [40]. Its protein product functions in the positive regulation of the

cell cycle. The levels of mRNA of c-Myc are regulated via the LIF signalling pathway by transactivation of c-Myc transcription by means of binding of phosphorylated STAT dimers to the c-Myc promoter region. Forced expression of stabilized c-Myc can sustain the pluripotent state of mESC in the absence of LIF [40]. Stabilization of c-Myc is most often achieved by targeted mutagenesis affecting the T58 codon in c-Myc, which is the target for GSK3β-dependent phosphorylation of c-Myc, resulting in its subsequent ubiquitination and degradation [41]. mESC expressing T58A mutant c-Myc sustain their stemness characteristics in LIF-free growth medium and in presence of mutated STAT3, which is devoid of transactivation properties. GSK3β is the beta isoform of the glycogen synthase kinase 3, a serine/threonin kinase phosphorylating target proteins such as p53, Axin, Notch, and SMAD3 (see below). Inhibition of GSK3β in mESC results in growth acceleration, producing tumour-like structures [42]. Inhibition of GSK3 together with inhibition of ERK in very early murine embryos supports derivation of 'naïve' mESC [43, 44].

The role c-Myc plays in the maintenance of the pluripotent state and the capability for self-renewal of mESC is likely to be implemented via more than one mechanism. Among these, prominent is the ability of c-Myc to inhibit endodermal differentiation by suppressing its crucial regulator, Gata6 [45], by stimulating the expression of the catalytic subunit of the telomerase complex (TERT) [46] and of microRNAs characteristic of the undifferentiated state [47].

STAT3 is a crucial factor in the maintenance of the undifferentiated state of naïve mESC. Its activation by means other than signalling through LIF may mimic the action of LIF in the maintenance of pluripotency in mESC. In order to maintain the undifferentiated state, however, besides LIF and/or STAT3, the presence of foetal serum is required, which indicates that there are additional diffusible factors that are needed to maintain self-renewal of mESC in culture [48]. Notably, STAT3 signalling is involved in self-renewal of naïve mESC but not primed mESC and hESC (see below) [43].

TGF-β signalling

As was previously mentioned, LIF alone is sufficient to ensure the maintenance of the pluripotent state of naïve mESC, provided that the growth medium contains foetal calf serum. If the medium is replaced with serum-free medium, however, the cells would spontaneously begin differentiation along the neuronal lineage, regardless of the presence or the absence of LIF. Obviously, the foetal serum contains one or more growth factors acting synergistically with LIF to maintain the pluripotent state

and to preserve the capacity for self-renewal. In 2003, Ying et al. identified the compound in the calf foetal serum partnering LIF in the prevention of induction of ESC differentiation and the maintenance of the pluripotent state – namely, the bone morphogenic proteins (BMP) 4 [49]. BMP is a collective term for a group of proteins related to the transforming growth factor β (TGF-β) but belonging to various families – growth factors and cytokines acting in cell proliferation, differentiation, programmed cell death, etc. These are secretable ligands that bind to heterodimers of transmembrane receptor tyrosine kinases type I and II [50]. Binding of BMP results in activation of the receptor complex, which in turn phosphorylates downstream the SMAD family of intracellular signalling proteins (Figure 2).

SMAD proteins are broadly classified into three large categories – R-SMAD (receptor – regulated SMAD); common-mediator SMAD (co-SMAD) and inhibitor SMAD-proteins (I-SMAD). Upon binding of BMP, receptor-regulated SMAD (SMAD1, SMAD5 and SMAD8 in mESC) are phosphorylated by the activated transmembrane tyrosine kinase and form a heterotrimeric
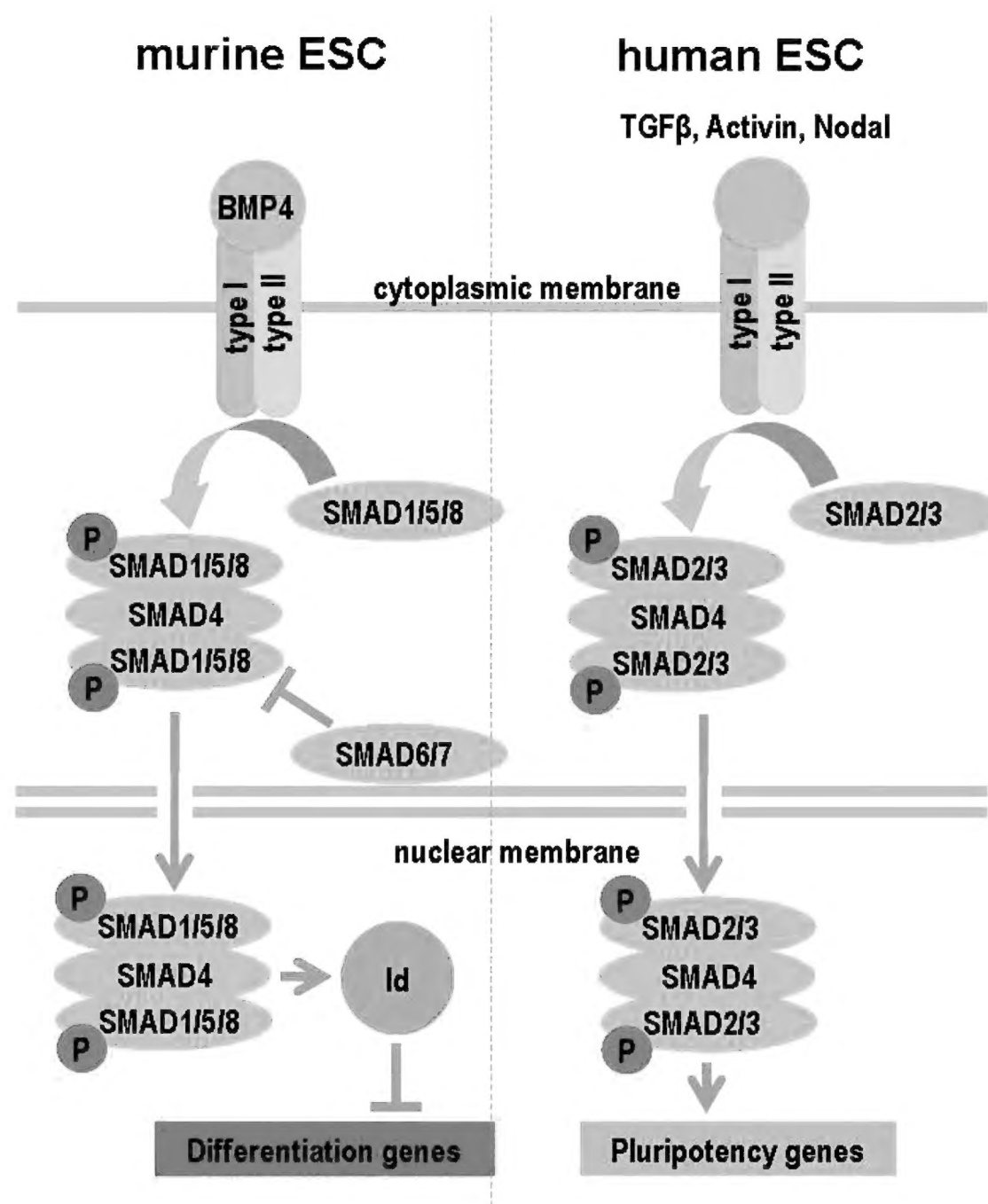

Figure 2. Signalling induced by growth factors of the TGF-β family in mESC and hESC. Curved arrows indicate phosphorylation; straight arrows indicate activation; ⊥-shaped line indicates inhibition.

complex with the only co-SMAD protein identified so far in mammals - SMAD4. The heterotrimer enters the nucleus where it functions as a transcription factor. The process is subject to negative regulation by inhibitor (I)-SMAD (SMAD6 and SMAD7). Namely, I-SMAD repress binding of R-SMAD to co-SMAD (SMAD4) by competing with SMAD1 for SMAD4, and by stimulating the degradation of the receptor kinases and R-SMAD via the ubiquitin-dependent pathway [51]. BMP4 stimulates the expression of the inhibitor of differentiation (Id) in naïve mESC, a transcription factor which suppresses the expression of many genes associated with cell differentiation, including differentiation along the neuronal lineage. Adding BMP4 to mESC grown in serum-free but LIF-supplemented growth medium halts the process of differentiation [52]. Forced expression of Id in naïve mESC enables them to sustain their undifferentiated state in growth medium supplemented only with LIF, without addition of BMP4 or serum. It must be noted, though, that mESC grown in LIF-free medium containing only BMP4 would again begin to differentiate, this time along the mesodermal lineage [53]. Apparently, the maintenance of the pluripotent state of mESC requires precise quantitative balance between the two factors.

hESC and primed mESC are also sensitive to the presence of BMP4 in the growth medium, but the mechanism seems to work in exactly the opposite manner – instead of maintaining the pluripotent state, BMP4 stimulates the differentiation of human ESC into trophectoderm or primitive endoderm [54]. In hESC, the functions required for the maintenance of the pluripotent state are implemented by other members of the TGF-β family. Among these prominent is, for example, Activin A, another TGF-β-like protein secreted by feeder fibroblasts. Activin A can be added as a supplement to growth media of hESC, allowing them to be maintained in undifferentiated state in the absence of feeder layer [55]. Signalling in hESC maintained into pluripotency *in vitro* is carried out via a TGF-β/Activin A/Nodal pathway similar to the BMP4-dependent pathway in mESC. As in the BMP4-dependent pathway, signal transduction is based on activation of the downstream R-SMAD (for hESC these are SMAD2 and SMAD3) [56] (Figure 2).

Wnt signalling

The Wnt protein family of ligands are glycoproteins rich in cysteine. Wnt proteins are known to play a role in three signalling pathways, one of which (canonical Wnt pathway) relays signals via β-catenin (Figure 3). The other two pathways, collectively known as non-
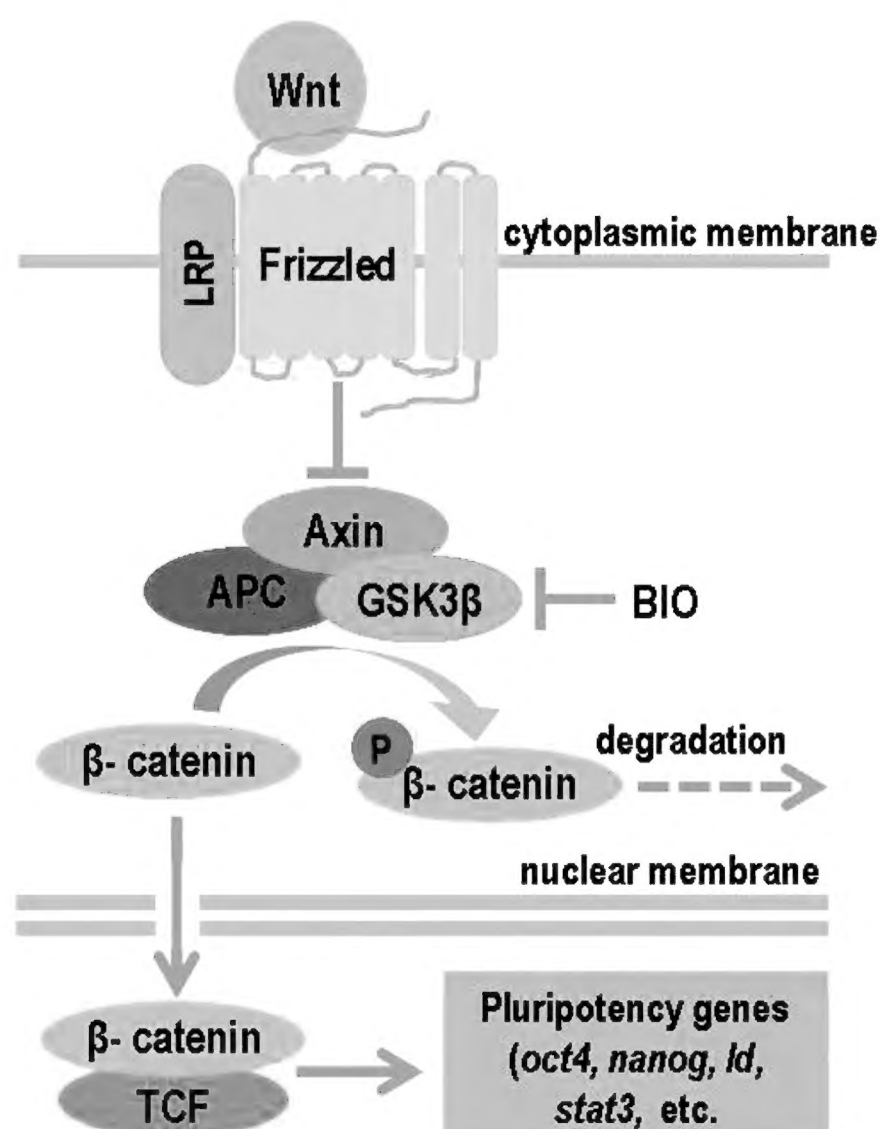

Figure 3. Canonical Wnt signalling in stem cells. Curved arrows indicate phosphorylation; straight arrows indicate activation; ⊥-shaped line indicates inhibition.

canonical Wnt-signalling, transmit signals from the cytoplasmic membrane to the nucleus via other molecules, such as the tyrosine kinase JNK; the small heterotrimeric G-proteins (small GTP-ases); and also Ca^{2+} [57].

The cytoplasmic protein β-catenin plays a basic role in canonic Wnt-signalling. β-catenin has a dual function, linking cadherin receptors to the actin cytoskeleton in neighbouring cells, thereby constituting an integral part of intercellular contacts, on the one hand and on the other hand acting as an intracellular messenger [58]. When there is no Wnt ligand present inside the cytoplasm, the unbound cytoplasmic β-catenin is phosphorylated by a complex consisting of APC - the protein product of the adenomatous polyposis coli gene; Axin (axis-inhibitor 1) and GSK3β. Phosphorylated β-catenin is marked for degradation via the ubiquitin-dependent pathway, thus ensuring that in the absence of the Wnt ligand the level of β-catenin is maintained low. In the presence of Wnt ligand inside the cell, however, β-catenin binds to its receptor proteins Frizzled (a 7-pass transmembrane protein) and LRP5/6 (low-density lipoprotein receptor-related 5 and 6, transmembrane 1-pass proteins) and via Axin/ LRP5/6 binding and/or activation of the Axin-binding cytoplasmic phosphoprotein Dishevelled causes inactivation of GSK3β. As a result, the degradation of β-catenin is suppressed, it accumulates in the cytoplasm and after reaching certain threshold level it translocates to the nucleus, where it transactivates the gene *TCF* (T-cell factor). The protein product of the *TCF* gene, in turn, stimulates the expression of a set of target genes, among which are the pluripotency genes *Oct4, Nanog, Id* and *Stat3* [58-60]. The Wnt signalling pathway *in vitro* can be activated by supplementing the growth medium with a specific GSK3β inhibitor commonly called BIO (6-bromoindirubine-3'-oxim). In the presence of BIO, the undifferentiated state of both mESC and hESC can be maintained *in vitro* [61]. Apparently, the different signalling pathways playing a role in the maintenance of the pluripotent state may cross and overlap, with regard to the different participants as well as with regard to their functions.

Signalling pathways mediated by PI3K/Akt

Phosphatidylinositol-3-kinases (PI3K) are a family of proteins with kinase activity, functioning as signal transmitters in cell signalling. PI3K catalyse the phosphorylation of the hydroxyl group in position 3 in the inositol ring of phosphatidylinositol and may be activated by various triggers. Such may be phosphorylation of PI3K by receptor tyrosine kinases

bound to their respective ligands (e.g. growth factors); or binding of regulatory subunits of PI3K class I to phosphorylated receptors. Activation of PI3K results in generation of second messengers such as phosphatidylinositol-3-phosphate (PI(3)P); phosphatidyl-inositol-(3,4)-bisphosphate $(PI(3,4)P_2)$ and phosphatidyl-inositol-(3,4,5)-trisphosphate $(PI(3,4,5)P_3)$. $PI(3,4)P_2$ and $PI(3,4,5)P_3$ bind to the pleckstrin homology domain of the serine/threonine kinases of the Akt family (protein kinases B, PKB) and to the P-domain of the phosphoinositide–dependent protein kinase 1 (PDPK1) and cause translocation of the Akt kinases to the cell membrane and their subsequent activation [62]. Activated Akt kinases regulate cell proliferation and suppress programmed cell death. The signalling pathway via PI3K/Akt is particularly important in maintaining the ability for self-renewal in ESC [63]. Inhibiting the activity of either PI3K or Akt results in initiation of differentiation of both mESC and hESC, regardless of the presence of LIF and/or of feeder layer. In particular, activation of the signalling pathways via Akt can maintain the pluripotency of mESC in a manner that is independent of the Wnt and LIF-related mechanisms, as it functions without affecting the cellular distribution of β-catenin or STAT3 activation [64].

One of the basic target molecules of Akt is mTOR (mammalian target of rapamycin). mTOR is a serine/threonine kinase which participates in the regulation of a plethora of cellular processes, among which are cell growth, division, apoptosis, motility, protein synthesis, etc. The activity of mTOR can be suppressed by adding rapamycin to the growth medium. This results in growth inhibition of ESC (that is, their self-renewal capacity), but does not trigger differentiation [65]. As could be expected, activation of mTOR is not sufficient to maintain the pluripotent state, unless LIF is also present. Apparently, the signalling component which directs the self-renewal via the Akt-dependent signalling pathway is not relayed via mTOR. Akt is capable, however, of accelerating the progress from G1 to S phase of the cell cycle or bypassing the G1/S checkpoint in pluripotent hESC even in the presence of DNA damage by means of stimulation of cyclin/CDK complex and regulation of cyclin D activity [66] (for details of the mode of action of the G1/S checkpoint in ESC, see below). It is likely that the signalling pathway via Akt functions to maintain the pluripotent state by inhibiting the mechanisms which act to suppress the progression through the cell cycle in the differentiated state. Forced expression of cyclin D alone, however, will not sustain the undifferentiated state of ESC [67]. Apparently, the molecular mechanism of maintenance of the undifferentiated state via the Akt-related signalling pathway is more complex than previously believed.

Signalling mediated by ERK1/2

The extracellular-signal-regulated kinases 1 and 2 (ERK1/2) are members of the family of the serine/threonine mitogen-activated protein kinases (MAPK). In most somatic cells MAPK are involved in the regulation of the progress through the early G1-phase of the cell cycle [68]. Depending on the properties of the activating stimulus and its duration, MAPK may reroute the cells either to differentiation or to self-renewal, which makes MAPK one of the important regulators of the cell fate.

The basic mechanism of activation of MAPK is associated with Ras – cellular proto-oncogene, member of the superfamily of small GTP-ases [69]. Ras is activated by binding of growth factors to their respective receptors, which induces formation of a complex between the adapter protein Grb2 and the G-nucleotide exchanging factor Sos. The latter induces the exchange of the Ras-bound GDP with GTP, which is a signal for Ras activation. The activated Ras-GTP initiates a cascade of transphosphorylation events together with another proto-oncogene, the serine/threonine kinase Raf. One of the phosphorylation targets is ERK1/2, which in its activated state phosphorylates numerous downstream substrates, and is translocated to the nucleus, where it activates several transcription factors directly involved in the positive regulation of cell division, such as c-Jun, c-Fos, Ets, and Elk [70]. Despite the fact that the Ras/ERK1/2-mediated pathway stimulates cell proliferation and that its induction increases the survival of many different cell types, the experimental data so far indicate that in mESC this signalling cascade may compromise the maintenance of the undifferentiated state and route the cell to differentiation. For example, forced expression of Ras in mESC results in mass differentiation along the endodermal lineage [71]. This finding triggered the development of a new set of strategies to maintain the undifferentiated state of mESC *in vitro* by adding inhibitors of the Ras/ERK1/2 signalling pathway to the growth medium, such as PD98059 [72-74]. As for the hESC, however, the signalling activated by growth factors (more specifically, the fibroblast growth factor FGF) may have different outcomes compared to mESC [75]. While the Ras/ERK1/2 signalling must be inhibited in naïve mESC in order to maintain the pluripotent state, primed mESC and hESC explicitly require supplementation of their growth medium with FGF in order to remain pluripotent [6]. Blocking of FGF receptors or the downstream ERK1/2-mediated signalling cascades in hESC results in differentiation into trophectoderm and primitive endoderm. In hESC, FGF is likely to work by induction of the expression of the pluripotency factors (OCT-4, NANOG, etc.). Supposedly, the induction could be carried out in direct (e.g. stimulation of transcription of the pluripotency genes) as well as in indirect manner (e.g. stimulating the feeder layer cells) [76, 77].

Features in rodent molecular physiology that makes mESC and hESC more unlike each other than expected

…the little mouse, how sagacious an animal it is, which never entrusts its life to one hole only; in as much as, if one hole is blocked up, it seeks another as a place of refuge.
Titus Maccius Plautus (c. 254–184 BC),
in: Truculentus, Act IV, scene 4

There are only few differences in the exogenous factors required to maintain the undifferentiated state of mESC and hESC. This is not unexpected, to say the least, as the mouse and the man share between 70 and 90 % similarity in their genomes [78], and the early stages in individual development are common for all mammals. Indeed, the factors which play a role in cell commitment and differentiation along a particular lineage may be different between mouse and man, but the basic stemness factors are essentially the same. There are differences, however, in how these factors function and what their effect on the pluripotent cell population is. These differences are often related to inter-species divergence, but also to some specific traits in rodent molecular physiology that make them unique in comparison to all other mammals not only in regard to the requirements to sustain the undifferentiated state, but also in basic molecular and cellular processes such as DNA repair, cell cycle, etc.

In the light of the current views of existence of two distinct states of pluripotent stem cells, it is believed that primate ESC (hESC included) exhibit properties more similar to primed (post-implantation) rodent ESC than to naïve (preimplantation) ESC [6, 24]. For example, in naïve mESC, both X-chromosomes of female embryos are activated while in primed mESC one X-chromosome is promptly inactivated, as in hESC. LIF/STAT3 signalling does not support the self-renewal of hESC and primed mESC, unlike naïve mESC in which LIF/STAT3 is a major mechanism for maintaining the ability to renew the stem cell population [43]. hESC express the signal-transmitting molecules LIFR, JAK and STAT3 at low levels, which appears to be insufficient for effective transmission of the signal. This may account, at least partially, for the fact that the maintenance of the pluripotency of hESC cannot be ensured by the presence of LIF and foetal serum only. Furthermore, hESC express at high levels the suppressor of cytokine signalling SOCS, which is known to inhibit the LIF signalling pathway [79]. In any case, however, LIF is dispensable in maintenance of pluripotent state of primed mESC and primate ESC, including hESC.

There are shared features between naïve mESC and primate ESC, too. For example, both hESC and primate ESC express markers which are not typical of primed mESC – REX1 being a prime example, and molecules typical for primed ESC such as FGF5 are not found neither in hESC nor in mESC [24]. It could be speculated that the mechanism for maintenance of pluripotency in hESC and in ESC from all non-human primates has at some point during evolution become independent of the LIF/STAT3 pathway [80].

Another key difference is in regard to the manner of managing DNA damage in the cells of the early embryo and, respectively, in ESC of murine or human origin. Since the ability to repair DNA is tightly linked to the ability of the cell to divide, it is obvious that management of DNA damage is crucial in cell survival and self-renewal. This is of particular importance in the cells of the early embryo, which are expected to divide quickly to form enough cells so as to lay the progenitors of all cell populations of the adult organism and all DNA-modifying events must be resolved before the cell proceeds to S phase. Embryonic cells are therefore exquisitely sensitive to the presence of DNA damage [81].

The cell cycle of ESC of all types is characterized by a shortened G1 phase compared to somatic cells, therefore, all checkpoint mechanisms designed to prevent damaged cells from entering the cell cycle are relatively relaxed, though to a different degree in different ESC types. Under *in vivo* and *in vitro* conditions the source of DNA damage, however, may be quite different. *In vivo*, the main potential sources of DNA damage in a dividing embryonic cell are mismatches produced by incorrect template copying (these are usually efficiently resolved by the system of mismatch repair) and oxidative stress produced by metabolism. Early embryos, however, live in conditions of relative hypoxia and rely on anaerobic glycolysis rather than on oxidative phosphorylation in order to obtain energy, so the amount of reactive oxygen species (ROS) produced by the cellular metabolism is lower than in somatic cells. *In vitro*, however, there might be more DNA damage to deal with, as ESC are often maintained for a long time in culture, and despite the fact that they age much more slowly than somatic cells, they do experience the cumulative effects of aging. Furthermore, ESC may be treated with various agents that may cause additional genotoxic stress (e.g. DMSO). In any case, there is a risk of genotoxic damage to embryonic cells, and there is not much choice, figuratively speaking, on how to proceed with damage resolution. The G1/S phase provides a major checkpoint in eukaryotic cells (also known as restriction point), and its strictness correlates with the potential risk of letting a cell carrying potentially harmful mutations to produce progeny [81-83]. In somatic cells, the presence of the restriction point is obligatory and failure to comply with

its requirements usually results in temporary or permanent cell cycle arrest or cell death via the apoptosis pathway. A somatic cell which have acquired the ability to bypass the restriction point of the cell cycle is usually well on the way to cancerous transformation. Embryonic cells, however, are programmed to operate under the restricted timeframe of early gestation, and damage-directed cell cycle arrest is not a viable option, as this would greatly reduce the chances for embryo survival. Mutagenesis is actively suppressed in embryonic cells compared to same-species somatic cells, sometimes in orders of magnitude [84, 85]. All this leaves out only one logical option - apoptosis of the damaged embryonic cells, in the hope that the intact cells may replenish the population. In some types of ESC (rodent ESC specifically) the R checkpoint is rendered inoperative, that is, cells with DNA damage may and do proceed with the cell cycle [86]. Some of these cells are diverted to the differentiation pathway, where the R checkpoint is active and can therefore implement the action necessary for damage resolution [87, 88], other, in which levels of DNA damage are assessed to be beyond a certain threshold, are eliminated by apoptosis. It has been found that p53 can suppress directly the expression of one of the basic pluripotency genes, *Nanog* [89, 90] and that the p53-dependent apoptotic response is enhanced similarly in mESC and hESC [81, 91, 92]. This way, rodent embryos who have sustained DNA damage have a chance to survive, though admittedly the resulting offspring may have low birth weight and congenital anomalies (due to premature and/or impaired differentiation), but since the embryos are usually more than one per pregnancy, at least some of them might survive.

The situation is somewhat different with human embryos. Human ESC are believed to have preserved their R checkpoint, though its efficiency is lower than in differentiated cells [93, 94], which means that replicative arrest induced by genotoxic stress might (and indeed does) reduce the chances for survival of affected embryos [95]. Some authors believe that this is one of the reasons why assisted reproduction using frozen gametes and embryos has lower than expected efficiency of producing pregnancy (up to 50 %, depending on the protocol), as freezing unavoidably includes treatment with agents capable of damaging DNA such as cryopreservatives; small molecules such as valproic acid, etc. [96-98]. Presence of the R checkpoint, even with low efficiency, would decrease both cell survival and the ability to self-renew the hESC population under conditions of genotoxic damage. Again, apoptosis of cells with damaged DNA is the only viable option, but this would inevitably result in a higher rate of gestation failure in embryos under genotoxic stress and in reduced viability of hESC in culture.

There is also the question of priority of repair in different genomic regions, as it is known that rodent cells

tend to route the NER-associated DNA repair machinery with priority to actively transcribed regions (a.k.a. rodent repairadox [99]). This effectively results in rodent cells being able to survive much larger amounts of some DNA-damaging agents than other mammal cells, human cells included; the latter attempt to repair all genomic lesions regardless of their location in actively transcribed or untranscribed genomic regions. The same mechanism has been found to operate in human cells that are in a permanent G0-arrest, such as the differentiated neurons and quiescent circulating B-lymphocytes [100-102]. It is not known whether this applies to rodent ESC also, especially considering that the chromatin of mammal stem cells is in hyperplastic state [103]; so it is more accessible to the repair machinery than in differentiated cells anyway. Similarly to all ESC, hESC exhibit a shortened G1 phase which would most likely be insufficient for methodical (and slow) repair of all DNA lesions. Therefore it would be unwise to proceed into the S phase of the cell cycle burdened with unrepaired DNA damage, especially since the repair machinery is not directed specifically towards the transcribed genes (as it presumably would in rodent ESC), meaning that the delicately balanced transcription profile of the undifferentiated state of hESC could be compromised at any time, possibly with a plethora of undesired effects. Just as mESC, human embryonic cells choose to remove damaged cell selectively by apoptosis. Since eliminating every cell that has any degree of DNA damage straight away would increase the risk of early gestation failure, as much as loss of proliferative capacity because of cell cycle arrest of damaged cells, hESC deploy the R checkpoint so as to allow for some degree of damage assessment before embarking on a decision for apoptosis. Both extremes carry the same inherent risk for loss of embryo, but there is still a considerable leeway, as DNA repair in embryonic cells is over-stimulated and may potentially be able to restore in time the normal state of the cellular DNA, thereby complying with the requirements of the restriction point in order to proceed normally to the S phase. Therefore, while mESC may rely on reprogramming the cell into differentiation route/apoptosis in order to combat consequences of DNA damage in the undifferentiated state, hESC are forced to rely on effectiveness of DNA damage detection in order

to proceed with DNA repair first and resort to apoptosis only if this mechanism fails. Coupled with species-specific physiological features, this arrangement seems to work well for both species, albeit in a different manner.

Conclusion

And when they reached their house, they found (besides their want of Stuffin',)The Mouse had fled; - and, previously, had eaten up the Muffin.
Edward Lear, in: "Laughable Lyrics" (1877)

Mice and rats are considered to be close enough to humans to be used as animal models in most research and applications eventually intended to be used in the field of human biomedical science, while being at the same time sufficiently distantly related to primates in aspect of phylogeny so as not to overstep ethical boundaries. This concept satisfies the requirements of many fields in modern biology and medicine, with several exceptions where data from mouse models cannot be translated directly into human research and therapy. Prominent among the latter are some areas of pharmacology, some types of nuclear transactions (e.g. DNA repair) and several aspects of stem cell science. All these can draw a very definite line between the mouse and the man. While basic factors and signalling mechanisms remain the same, they can work in a very different matter in the two species, producing different outcomes. Studying the specific molecular features of both species in the specific context of maintenance of the undifferentiated state of stem cells can provide researchers with the unique opportunity to unravel the complex network of interactions which takes part in the decision of cell fate under different conditions, to glean interesting insights into the parallel evolution of the two species and to observe how different variants of basic cellular processes have been tried and tested in the evolutionary process.

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